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Letters

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An Engineered Allele of *afsQ1* Facilitates the Discovery and Investigation of Cryptic Natural Products

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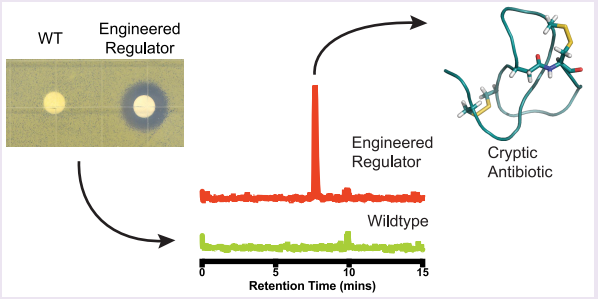
Supporting Information

ABSTRACT: New approaches to antimicrobial discovery are needed to address the growing threat of antibiotic resistance. The *Streptomyces* genus, a proven source of antibiotics, is recognized as having a large reservoir of untapped secondary metabolic genes, many of which are likely to produce uncharacterized compounds. However, most of these compounds are currently inaccessible, as they are not expressed under standard laboratory conditions. Here, we present a novel methodology for activating these "cryptic" metabolites by heterologously expressing a constitutively active pleiotropic regulator. By screening wild *Streptomyces* isolates, we identified the antibiotic siamycin-I, a lasso peptide that we show is active against multidrug pathogens. We further revealed that siamycin-I interferes with cell wall integrity *via* lipid II. This new technology has the potential to be broadly applied for use in the discovery of additional "cryptic" metabolites.



A characteristic of the *Streptomyces* genus of Gram-positive soil bacteria is its ability to produce a multitude of bioactive natural products. These compounds have anticancer, immunosuppressive, anthelmintic, and antifungal activities, although most commonly these molecules are antibacterial. Indeed, the vast majority of clinically relevant antibiotics originate from this genus.[1] Since the 1970s, however, the rate of discovery of new antibiotics from this source has decreased steadily. Ironically, genome sequencing has revealed that all *Streptomyces* have far greater biosynthetic capacity than had been appreciated originally: biosynthetic gene clusters for 25 or more secondary or specialized metabolites are encoded in virtually every streptomycete genome, with the total metabolic diversity unknown, but certainly very large.[2] Many of these gene clusters are predicted to generate unknown compounds, suggesting that a majority of the natural products in this genus' armamentarium remains to be discovered. It appears, however, that many of the gene clusters are "cryptic" in that they are expressed at very low levels, or not at all in the laboratory. Developing methods for inducing their biosynthesis such that these unknown molecules can be purified and studied is therefore of significant interest.[3−7]

Genetic studies have revealed many pleiotropic regulators that influence the production of multiple secondary metabolites in *Streptomyces*.[8] One of these regulators is the *afsQ* two-component system (Figure 1a). In *Streptomyces coelicolor*, the histidine kinase AfsQ2 autophosphorylates in response to a signal that may be related to nitrogen metabolism and then transfers the phosphoryl group to Asp52 on AfsQ1. This induces a conformational change allowing AfsQ1 to bind to DNA and positively regulate many genes involved in the production of secondary metabolites.[9−11]

The *afsQ* genes are widely conserved in the streptomycetes. Assuming that these genes regulate secondary metabolism in other *Streptomyces* species, and given their near ubiquity, we reasoned that the AfsQ1 transcription factor could be used as a tool for activating cryptic secondary metabolic genes. Indeed, our previous work and others using different transcriptional regulators support this strategy.[12,13]

We generated an activated allele of the *S. coelicolor afsQ1* that we predicted would bypass the need for phosphorylation by AfsQ2 and any knowledge of the specific signal needed to modulate AfsQ2 activity. We show that expressing this allele activates diverse secondary metabolites in multiple *Streptomyces* strains. We successfully purified a cryptic antibiotic from WAC00263 (a wild *Streptomyces* isolate) and established both its antibacterial activity and its mode of action. Notably, it is active against a variety of drug-resistant Gram-positive pathogens and targets cell wall biosynthesis, likely *via* the

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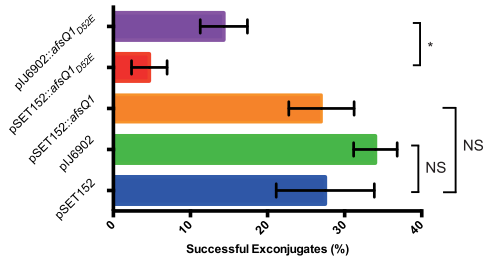
A)

B)

Representative Genomes With AfsQ1 Homologue	Representative Genomes Without AfsQ1 Homologue
S. albus	*S. flavovariabilis*
S. avermitilis	*S. halstedii*
S. coelicolor	*S. novaecaesareae*
S. griseus	
+ 87 other Genomes	

Representative Genomes With AfsQ2 Homologue	Representative Genome Without AfsQ2 Homologue
S. albus	*S. alboflavus*
S. avermitilis	*S. avellaneus*
S. coelicolor	*S. flavovariabilis*
S. griseus	*S. halstedii*
S. nodosus	*S. himastatinicus*
S. scabiei	*S. novaecaesareae*
+79 other genomes	*S. olivaceus*
	S. pratensis
	S. yeochonensis

C)



D)

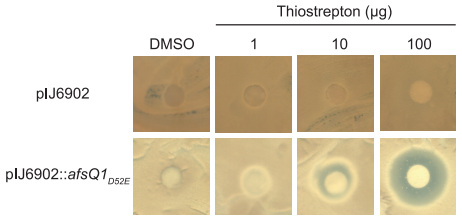


Figure 1. Overexpression of AfsQ1$_{D52E}$, lethal and resulting in low conjugation efficiency. The AfsQ operon (A) consisting of the response regulator AfsQ1 histidine kinase AfsQ2, putative lipoprotein AfsQ3, and the sigma factor SigQ is highly conserved. (B) Representative *Streptomyces* genomes from the NCBI lacking an obvious AfsQ1/2 orthologue (based on reciprocal BLAST searches to the *S. coelicolor* genome) are listed. (C) Conjugations with 47 wild *Streptomyces* isolates showed that constitutive, high-level expression AfsQ1$_{D52E}$ led to a significant decrease in the number of successful exconjugates, while conjugations with empty vector and the vector carrying the wildtype gene (not overexpressed) showed no differences. Conditional expression increased the number of recoverable exconjugates. Data represent the result of three independent series of conjugations. Error bars: ± standard deviation. Conjugations were considered significantly different if an unpaired Student test gave a *P* value <0.05(*). NS = not significant. (D) Induction of *afsQ1*$_{D52E}$ with thiostrepton in *S. lividans* yielded a zone of inhibition suggesting overexpression was lethal; induction of a plasmid-alone control (pIJ6902) with thiostrepton had no effect. Amount of thiostrepton on each disk indicated above.

essential precursor lipid II. We suggest that this is a widely applicable approach to identifying and studying low abundance natural products.

Heterologous Expression of Constitutively Active *afsQ1* in Wild Isolates. We identified probable *afsQ1* and *afsQ2* orthologues in nearly all *Streptomyces* genomes: 97% of species in the NCBI repository encode both the response regulator and histidine kinase (Figure 1b). To construct a constitutive allele of *afsQ1*, we generated a phosphomimetic mutant that altered aspartate 52, the presumed phosphorylation site, to a glutamic acid generating the gene *afsQ1*$_{D52E}$. We placed this allele under the control of the constitutive *ermE** promoter in the integrating pSET152 vector. While preliminary experiments (not shown) suggested that this construct induced cryptic secondary metabolites in some strains, it was lethal in most. For example, while the empty vector was successfully introduced into 11 of 47 wild isolates, the *afsQ1*$_{D52E}$ expressing construct could only be stably introduced into three (Figure 1c). The wildtype gene behaved as the empty vector suggesting that the lethality was due to the D52E gain-of-function mutation.

To overcome this limitation, we placed *afsQ1*$_{D52E}$ under the control of a thiostrepton-inducible promoter. This increased the number of strains that could incorporate *afsQ1*$_{D52E}$ by 3 times (Figure 1c). Titrating thiostrepton in this expression system demonstrated the lethality associated with expression of *afsQ1*$_{D52E}$. In *S. lividans*, thiostrepton induction led to a zone of inhibition conditional on the expression of *afsQ1*$_{D52E}$, whereas the control strain showed no inhibition (Figure 1d).

To test this system for the induction of natural products, we grew the engineered strains with the *afsQ1*$_{D52E}$ allele in the absence and presence of thiostrepton. We examined control and induced crude extracts using LC-MS and found that *afsQ1*$_{D52E}$ activated the production of cryptic secondary metabolites in many strains. For example, in *S. aureofaciens*, a known chlorotetracycline producer, four unknown cryptic metabolites were induced by *afsQ1*$_{D52E}$, while chlorotetracycline production remained unchanged (Figure 2a–d and Supplemental Figure 1). Three of these compounds (m/z = 720.39, m/z = 773.42, m/z = 759.41 $[M + H]^+$) were produced in small quantities in the control strain, and expression of *afsQ1*$_{D52E}$ elevated yields by 10–100 fold. At least one compound, m/z 893.44, was entirely absent without thiostrepton-induced *afsQ1*$_{D52E}$. From the accurate mass data, we generated possible chemical formulas for each of these metabolites but none could be attributed to known metabolites from *S. aureofaciens*. These data confirmed that *afsQ1*$_{D52E}$ could be used to induce or elevate cryptic metabolite production.

Heterologous Expression of Constitutively Active *afsQ1* Induces Antimicrobial Activity. We examined crude extracts of three wild *Streptomyces* isolates and found that an extract from strain, WAC00263 exhibited antimicrobial activity against *Micrococcus luteus* and *Bacillus subtilis* contingent on the expression of *afsQ1*$_{D52E}$. This suggested that the mutant

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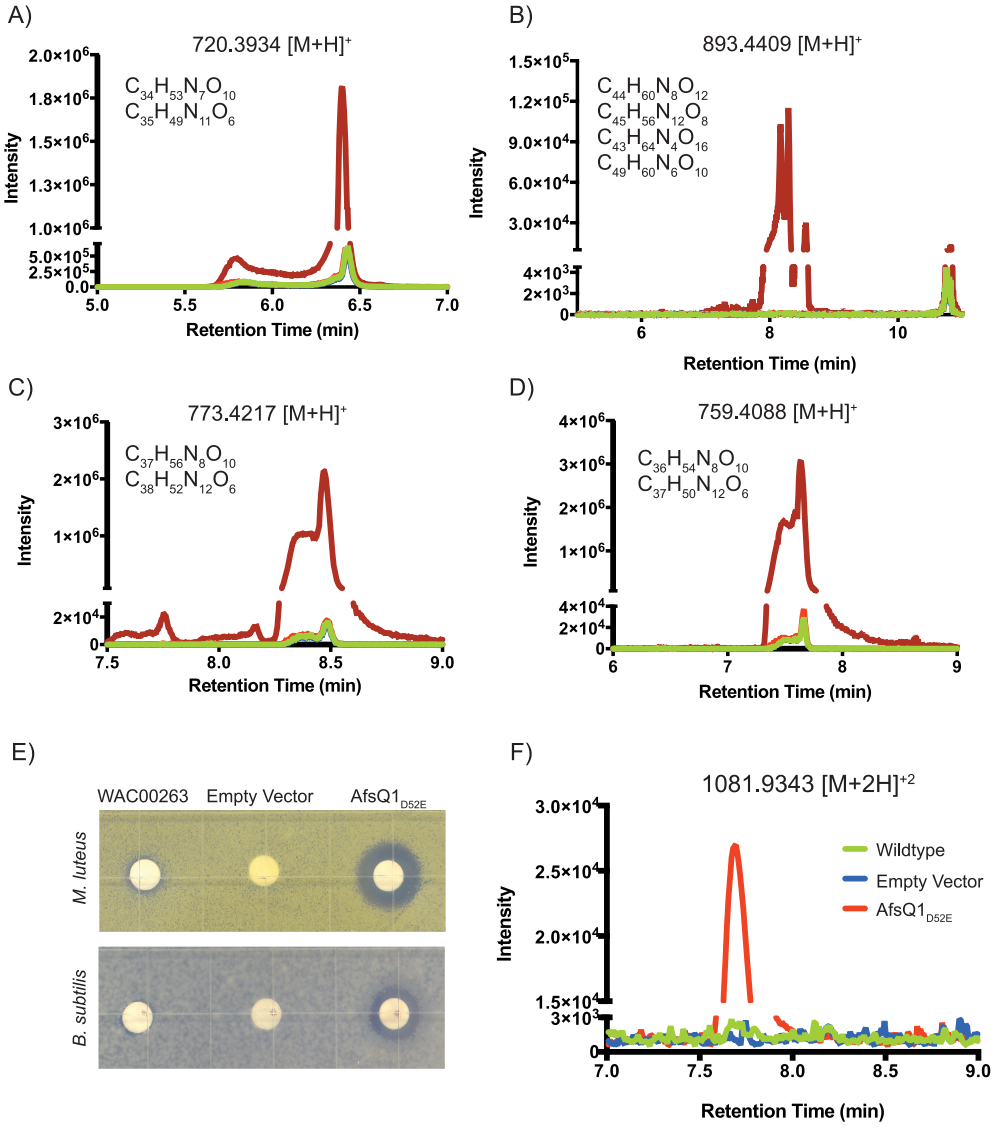

Figure 2. Overexpression of constitutively active AfsQ1 inducing production of cryptic metabolites and antimicrobial activity. (A–D) Extracted ion chromatograms (ion labeled above graph) from a metabolic profile of *S. aureofaciens* + $AfsQ1_{D52E}$ butanol extracts supplemented with (red) and without (orange) 10 μg/mL thiostrepton reveals induction of four cryptic metabolites. Empty vector control is shown in green. Chemical formulas generated with error <5 ppm. (E) Butanol extracts of wildtype WAC00263 and extracts from the strain containing the empty vector and $afsQ1_{D52E}$ allele were spotted onto disks to monitor antimicrobial activity against *M. luteus* and *B. subtilis*. The zone of inhibition observed relied upon the expression of $afsQ1_{D52E}$. (F) Comparing the metabolomic profile of extracts revealed production of a "cryptic" metabolite. Extracted ion chromatograms of metabolite m/z 1081.9343 for each sample are shown. Production of the metabolite is contingent on expression of $afsQ1_{D52E}$ (red), as expression of the wildtype gene (green) or empty vector (blue) shows no production.

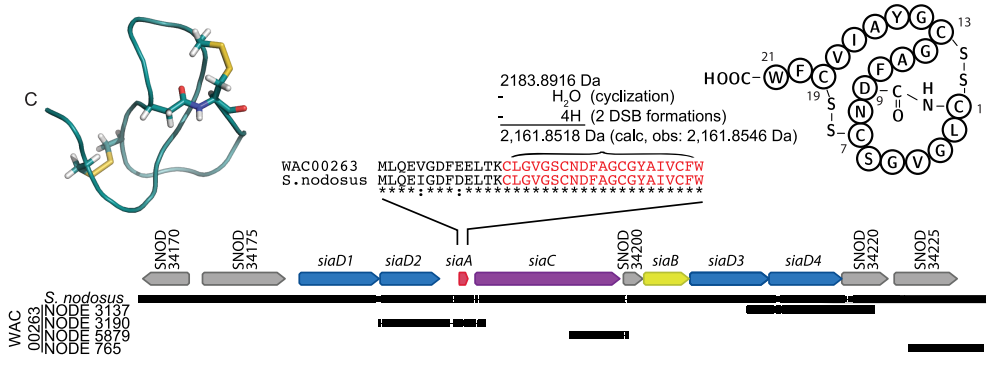

Figure 3. Schematic of the WAC00263 siamycin-I biosynthetic gene cluster and alignment with the homologous genes in *S. nodosus*. Amino acids highlighted in red represent the primary structure of the metabolite, which is knotted to form siamycin-I as shown.

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A)

Strain Name	ATCC#	Clinic Resistance	MIC (μM)
M. luteus			0.9
B. subtilis			1.9
S. aureus	29213		3.7
S. aureus	BAA-44	MRSA	7.4
S. aureus	BAA-41	Multi-Drug	14.8
S. epidermidis	12228		1.9
S. saprophyticus	15305		29.6
E. faecalis	29212		3.7
E. faecalis	51299	VRE	7.4

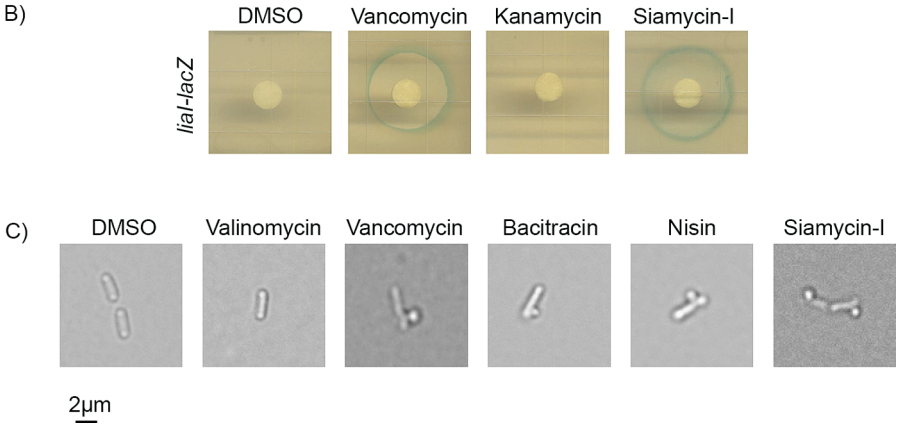

Figure 4. Siamycin-I influence on *B. subtilis* cell wall integrity likely through lipid II. (A) Siamycin-I is active against several Gram-positive bacteria with their MICs listed. (B) Transcriptional *liaI* reporter in *B. subtilis* was activated when challenged with siamycin-I and the positive control vancomycin. (C) Light microscopy confirmed that cell wall integrity was impaired, as membrane blebs were observed following siamycin-I treatment.

gene had induced the production of a cryptic antibiotic (Figure 2e).

To identify $afsQ1_{D52E}$ induced compounds, we compared extracts of the wildtype, empty vector and $afsQ1_{D52E}$-expressing strains using LC-MS. We identified two metabolites with masses of 2161.85 and 781.32 Da that were only produced with the expression of $afsQ1_{D52E}$ and not in either control strain (Figure 2f and Supplemental Figure 2).

We purified both induced metabolites. Testing their MICs against *M. luteus* by broth dilution revealed that both molecules had antibacterial activity. The 781.32 Da compound was of interest because there is no related mass in either the *Dictionary of Natural Products* or *Antibase*. However, the 2161.85 Da metabolite was more potent than the 781.32 Da metabolite, so we focused our efforts on this compound.

Identification of the Cryptic Antibiotic Siamycin-I and Associated Biosynthetic Cluster. Considering the size and UV absorbance profile of the metabolite, we hypothesized that it was a ribosomally synthesized natural product. We sequenced the genome of WAC00263 using illumina sequencing and identified small open reading frames that could translate peptides <10 000 Da. These protein sequences were then compared against the MS/MS fragmentation pattern of our unknown metabolite. The only match for the 2161.85 Da antimicrobial compound was the class-I lasso peptide siamycin-I (Figure 3 and Supplemental Figure 3). Siamycin-I consists of 21 amino acid residues including an N-terminal macrolactam resulting from an isopeptide bond between the N-terminus and an aspartate side chain at position 9. This lactam formation traps the C-terminal tail within the macrocycle and is stabilized on either side by two disulfide bonds as shown in the ribbon diagram derived by NMR of a siamycin-I isomer.[14]

To identify the entire biosynthetic cluster of siamycin-I, we searched for orthologues of the siamycin-I pro-peptide (*siaA*) in the NCBI database. The only sequenced genome encoding a *siaA* orthologue was *S. nodosus*. This organism is the only known producer of the antifungal amphotericin B and is not known to actively produce siamycin-I.[15] We used the *S. nodosus* genome to scaffold the contigs assembled from our WAC00263 illumina data. The resulting aligned contigs from WAC00263 shared ≥70% nucleotide identity with the siamycin-I cluster in the *S. nodosus* genome. The DNA surrounding the *siaA* pro-peptide-encoding gene revealed all the genes necessary for the assembly and export of a lasso peptide (Figure 3 and Supplemental Table 3). Downstream of *siaA* was a predicted asparagine synthase, *siaC*, hypothesized to form the hallmark isopeptide bond. The cluster further contained a trans-glutaminase-like protease encoding gene, *siaB*, necessary for cleaving the leader sequence of SiaA and releasing the folded metabolite. The cluster also encoded ABC transport proteins likely to mediate the export of the processed metabolite. Adjacent to these core genes we identified genes for a histidine kinase (SNOD34175) and a DNA-binding response regulator (SNOD34170) that potentially is involved in regulating siamycin-I production. This is the first report of the complete siamycin-I biosynthetic cluster, and it shares some similarity

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with the only other class I lasso peptide SSV-2083 from *S. sviceus*.[16] This new set of genes can now be utilized for bioinformatic exploration for new lasso peptides of this specific class.

Siamycin-I is Potent Antibacterial Compound. Importantly, we found that induction of $afsQ1_{D52E}$ led to sufficiently robust siamycin-I production by strain WAC00263 that the compound could be easily purified for activity and mechanistic investigations. The engineered strain produced yields of ∼15 mg of pure siamycin-I per 1 L of culture.

Several biological activities have been suggested previously for siamycin-I. It was originally identified as an inhibitor of HIV-induced syncytium formation.[17,18] It also blocked replication of a primary HIV-1 isolate.[18] Later studies demonstrated a specific interaction with the HIV envelope glycoprotein, gp160.[19] In an unrelated screen, it has also been shown that siamycin-I can inhibit myosin light chain kinase isolated from chick gizzards.[20] During investigations of siamycin-I's eukaryotic bioactivities, some antibacterial activates are presented against a limited number of bacteria, but deeper understanding of its antibacterial mechanism in bacteria is never addressed.[17,18,20] Interestingly, in *Enterococcus faecalis*, siamycin-I was shown to inhibit the autophosphorylation by the FsrC sensor kinase, resulting in the attenuating biofilm formation.[21,22] This still does not account for siamycin-I's antibacterial activity.

Using disk diffusion assays, we found that siamycin-I had activity against Gram-positive but not Gram-negative bacteria. For example, we observed potent activity against *M. luteus*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Staphylococcus saprophyticus*, and *Enterococcus* but not *Escherichia coli*, *Burkholderia cepacia*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, or *Klebsiella pneumoniae*. We furthermore found no bioactivity against the eukaryotic microbes *Saccharomyces cereviciae*, *Candida albicans*, *Cryptococcus neoformans*, or *Cryptococcus parapsilosis*. Our results suggested that siamycin-I was an antibacterial compound having activity limited to Gram-positive bacteria, which is consistent with earlier studies.[17,18,20]

Intriguingly, we found that siamycin-I has potent antimicrobial activity against both methicillin-resistant *S. auereus* (MRSA) and vancomycin-resistant *Enterococcus* (VRE) with an MIC of 7.4 μM (Figure 4a). Given that the compound is known to have limited cytotoxicity against human cells, it may be of clinical interest.[18]

Siamycin-I Targets the Cell Wall. To interrogate the mode of action of siamycin-I, we generated siamycin-I resistant *S. aureus* strains by serial passage in the presence of siamycin-I, up to 16 times the MIC. In addition to siamycin-I resistance, these strains also exhibited dramatic resistance to the peptide antibiotic nisin (MIC ≥ 24 μg/mL from 5 μg/mL) as well as moderately (2-fold) increased resistance to bacitracin and vancomycin (Supplementary Table 4). In contrast, all of the resistant strains exhibited the same sensitivity to kanamycin, a protein synthesis inhibitor, as their parent. The siamycin-I resistance profile therefore suggested a cell wall target. Moreover, this resistance pattern implicates lipid II, an essential cell wall precursor given that the observed cross-resistance with nisin, vancomycin, and bacitracin are all lipid II antagonists.

Pursuing the hypothesis that siamycin-I interferes with cell wall synthesis through lipid II, we examined cell envelope stress in *B. subtilis*. Interfering with the lipid II cycle upregulates the two component system *liaRS* through the identified promoter named *liaI*.[23] To determine whether this stress response was activated by siamycin-I, we treated a *B. subtilis* strain harboring a *liaI-β-gal* promoter fusion with siamycin-I and various control antibiotics (Figure 4b). Vancomycin treated cells produced the expected blue ring at the edge of the zone of inhibition, demonstrating activation of the *liaI* promoter. Cells treated with siamycin-I also exhibited activation of the *liaI* reporter, confirming that the antibiotic acts through the cell envelope and possibly lipid II. In contrast, kanamycin exhibited antibacterial activity but no activation of the *liaI* reporter.

A hallmark of antibiotics that act *via* lipid II is the formation of membrane bulges and blebs in methanol−acetic-acid-fixed cells.[24] This is due to the extrusion of the cell membrane through perforations that develop in the peptidoglycan layer at sites of active cell wall synthesis, where the action of lipid II antagonists is most evident. To determine whether siamycin-I had this effect, we used light microscopy to compare its phenotypic effects with those of other antibiotics.

As expected, *B. subtilis* cells treated with nisin, vancomycin, and bacitracin, all of which alter lipid II function, exhibited large blebs consistent with sites of compromised peptidoglycan production. Siamycin-I treated cells exhibited an identical morphology. In contrast, cells treated with valinomycin (which acts on the cell wall, but as an ionophore rather than a lipid II antagonist) did not exhibit this phenotype (Figure 4c). These data demonstrate that siamycin-I acts *via* a mechanism related to that of nisin, vancomycin, and bacitracin, likely involving interaction with an enzyme or intermediate in the lipid II biosynthetic pathway. There has been a renewed interest in lipid II antagonists spurred on by the discovery of texiobactin.[25] It is an attractive antimicrobial target for a variety of reasons, including its localization in the outer cytoplasmic membrane, its confinement to the bacterial kingdom, and its rate limiting activity in cell wall biosynthesis.[26]

In sum, we report an inducible, modified AfsQ1 allele with the potential to be used as a novel and widely applicable tool for triggering cryptic secondary metabolite production in the streptomycetes. We have shown that this allele can induce high level production of cryptic metabolites, in turn facilitating biochemical and biological characterization of these compounds.

METHODS

Generation of $afsQ1_{D52E}$ Expression Systems. Plasmids, strains, and primers can be found in the Supporting Information. Wildtype *afsQ1* amplified from *S. coelicolor* M145 was cloned into *Bam*HI and NotI sites in the pSET152 vector, downstream of the *ermE** constitutive promoter. The mutant allele $afsQ1_{D52E}$ was synthesized and inserted into the same sites as in the wildtype gene. Additionally, $afsQ1_{D52E}$ was inserted into the NdeI and *Kpn*I sites of the pIJ6902 plasmid, downstream of the *tipA* promoter for conditional expression.

Medium-Throughput Conjugation. Conjugations were conducted in parallel fashion on 96-well plates containing a solid MS medium.[27] These plates were inoculated with both spores from *Streptomyces* strains grown on solid MYM media, and the conjugation-proficient *E. coli* ET12567/pUZ8002 donor strain carrying the different pSET152/pIJ6902 constructs. Following 12−14 h of incubation at 30 °C, wells were overlaid with a 30 μL of solution of 125 μg mL^{-1} nalidixic acid and 313 μg mL^{-1} apramycin. Plates were incubated for a further 1−2 weeks before exconjugates were pinned onto MYM media supplemented with apramycin and nalidixic acid. Conjugations were conducted in triplicate.

Comparative Metabolomics. Cultures were grown on solid MYM medium for 6 days at 30 °C. Cells and agar were then cut into small pieces, extracted in an equal volume of n-butanol, sonicated for 5 min, and left to macerate overnight. Crude extracts were then filtered

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through Whatman paper, evaporated to dryness, and resuspended in MeCN and water (1:1, v/v). Metabolomic analyses of the WAC00263 variants was performed on an Agilent 1200 series LC system coupled to a Bruker micrOTOF II with an electrospray ionization source. LC used a Kinetex C18 column (2.1 × 50 mm, 2.6 μm, 100 Å, Phenomenex), solvents H_2O with 0.1% formic acid (A) and and MeCN with 0.1% formic acid (B), a 40 °C column temperature, a flow rate of 0.2 mL/min, and the following gradient: 0.5 min 5% B, 5−9 min 95% B, 10−15 min 5% B. Metabolomic analysis for *S. aureofaciens* was performed on a Acquity I-Class UPLC coupled to a Waters Xevo G2-S QTof with an electrospray ionization source. LC used a Kinetex Biphenyl column (2.1 × 100 mm, 1.7 μm, 100 Å, Phenomenex), solvents H_2O with 0.1% formic acid (A) and MeCN with 0.1% formic acid (B), a 40 °C column temperature, a flow rate of 0.4 mL min^{-1}, and the following gradient: 25 min 5−95% B, 25−27 min 95% B, 28−30 min 5% B. Data were processed using mzMine, and two biological replicates were completed for each analysis.

Genome Sequencing of WAC00263. Genomic DNA was prepared by inoculating MYM medium with WAC00263; glass beads were added to the culture to improve agitation. After 2 days of incubation at 30 °C, genomic DNA was isolated using the DNeasy Blood and Tissue kit (Qiagen). The DNA library was prepared using the Nextera XT DNA Library (Illumina) and sequenced on a MiSeq instrument at McMaster's Farncome genomic facility. A draft genome was prepared using velvet.[28]

Isolation and Elucidation of Siamycin-I. WAC00263 harboring the constitutively expressed $AfsQ1_{D52E}$ was grown on solid MYM medium for 6 days at 30 °C. Cultures were then macerated in *n*-butanol, sonicated in a water bath for 15 min, and left to extract overnight. This extraction was performed twice and was dried using a rotary evaporator at 40 °C. Crude extracts were resuspended in MeCN−water (1:1, v/v) and separated using a C18 solid phase cartridge (Waters) with incremental step washes of increasing MeCN. The fraction that eluted with 50% MeCN and water was collected, dried, and injected on an Alliance 2695 HPLC (Waters) using a XSelect C18 column (10 × 100 mm, 5 μM, Waters) running an isocratic gradient of MeCN and water (1:1, v/v) + 0.1% formic acid at 5 mL min^{-1} at 35 °C. Fragmentation data for the purified compound were acquired using a Thermo Q-Exactive quadrupole-Orbitrap MS, and peptide identification was validated with mMass.[29]

Identification of Siamycin-I Biosynthetic Cluster. To identify the biosynthetic cluster, the peptide sequence of siamycin-I was aligned with predicted protein (<10 kDa) sequences within the WAC00263 genome using BLAST. This led to the identification of a small ORF in a short contig. To expand the cluster, the nucleotide sequence of the identified pro-peptide was aligned with all the *Streptomyces* genomes on the NCBI. This allowed the scaffolding of the WAC00263 contigs onto the complete cluster from *S. nodosus* (Accession: CP009313).

Antimicrobial Assays. LB plates were inoculated with noted indicator strain with sufficient cell density to produce a lawn of growth. Disks with impregnated listed antibiotics were placed atop the bacterial lawn, and plates were grown at 37 or 30 °C depending on the strain. To measure the MIC of the compound, indicator strains were grown overnight, subsequently subcultured 1/10 000. Diluted cells were then supplemented with the noted antibiotics and grown at 37 °C for 21 h, and turbidity was measured at 600 nm. MIC was determined from three biological replicates.

Developing Siamycin-I Resistant Mutants. To generate mutants resistant to siamycin-I, *S. aureus* was serially passaged in LB at 37 °C. Each day, strains were subcultured 1/1000 into fresh LB media supplemented with siamycin-I. Initially, siamycin-I was added at 1/16th the wildtype strain's MIC; this was then doubled with each consecutive passage up to 16 times the MIC. Three nonclonal mutants were isolated. In parallel, the parental strain was passaged with DMSO in place of siamycin-I as a control.

Cell Envelope lial Stress Response. To monitor cell envelope stress, the *IiaI* reporter strain was grown overnight in LB at 37 °C with 1 μg mL^{-1} erythromycin.[30] Following a 1/1000 subculture, this reporter strain was inoculated on an LB agar plate supplemented with 40 μg mL^{-1} X-gal sufficient for growth of a lawn. After allowing the plate to dry, disks with antibiotics were added and were incubated at 37 °C. Plates were visualized after 21 h.

Bacterial Cell Wall Integrity Assay. This method was adapted from previous work.[24] *B. subtilis* cells were grown in LB to an OD_{600} of 0.4 and aliquoted into separate vials. Cells were treated with either 4 μM siamycin-I, 2 μM vancomycin, 2 μg mL^{-1} valinomycin, 3 μg mL^{-1} bacitracin, 0.5 μg mL^{-1} nisin, or DMSO and were then incubated at 37 °C with shaking. After 15 min of compound exposure, 200 μL of cells were fixed in 1 mL of 1:3 acetic acid to methanol and left for 10 min at RT. Cells were then immobilized by adding 5 μL of the fixation reaction to 5 μL of warm LB with 0.5% agarose on a microscope slide. Imaging was performed on a Zeiss Axio Scope A1 wide-field microscope with a 100× oil immersion objective.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.6b01002.

Supplemental Tables 1−4 and Figures 1−3 (PDF)

Accession Codes

Genome sequence of WAC00263 was deposited into the NCBI, user accession number MPHV00000000.

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Notes

The authors declare no competing financial interest.

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